Reference: *Biol. Bull.* **202:** 204–212. (June 2002)

Characterization of Limb Autotomy Factor–Proecdysis (LAF_{pro}), Isolated From Limb Regenerates, That Suspends Molting in the Land Crab *Gecarcinus lateralis*

XIAOLI YU[1], ERNEST S. CHANG[2], AND DONALD L. MYKLES[1,*]

[1]*Department of Biology, Cell and Molecular Biology Program, and Program in Molecular, Cellular and Integrative Neurosciences, Colorado State University, Fort Collins, Colorado 80523; and* [2]*University of California, Davis, Bodega Marine Laboratory, Bodega Bay, California 94923*

Abstract. Molting and limb regeneration are tightly coupled processes, both of which are regulated by ecdysteroid hormone synthesized and secreted by the Y-organs. Regeneration of lost appendages can affect the timing and duration of the proecdysial, or premolt, stage of the molt cycle. Autotomy of all eight walking legs induces precocious molts in various decapod crustacean species. In the land crab *Gecarcinus lateralis*, autotomy of a partially regenerated limb bud before a critical period during proecdysis (regeneration index <17) delays molting so that a secondary limb bud (2° LB) forms and the animal molts with a complete set of walking legs. It is hypothesized that 2° LBs secrete a factor, termed limb autotomy factor–proecdysis (LAF_{pro}), that inhibits molting by suppressing the Y-organs from secreting ecdysone. Molting was induced by autotomy of eight walking legs; autotomy of primary (1°) LBs reduced the level of ecdysteroid hormone in the hemolymph 73% by one week after limb bud autotomy (LBA). Injection of extracts from 2° LBs, but not 1° LBs, inhibited 1° LB growth in proecdysial animals, thus having the same effect on molting as LBA. The inhibitory activity in 2° LB extracts was stable after boiling in water for 15 min, but was destroyed by boiling 15 min in 0.1 N acetic acid or incubation with proteinase K. These results support the hypothesis that LAF_{pro} is a peptide that resembles a molt-inhibiting hormone.

Introduction

In response to injury, decapod crustaceans autotomize walking legs and claws, which are regenerated before the next molt (reviewed in Skinner, 1985; Hopkins, 1993, 2001). Limb regeneration is composed of two phases. The basal growth phase, which can occur at any time during the molt cycle, involves primarily cell proliferation and differentiation, and it results in the formation of basal papillae. The proecdysial growth phase, which is restricted to the proecdysial or premolt period (mostly stage D_0), involves tissue growth and is under hormonal control (Holland and Skinner, 1976; Hopkins, 1993, 2001). In the fiddler crab, exposure to the molting hormone 20-hydroxyecdysone (20E) is essential for the proecdysial growth phase (Hopkins, 1989). In addition, a limb growth inhibitory factor (LGIF) has been shown to inhibit rapid proecdysial growth of limb bud in the land crab (Hopkins *et al.*, 1979). Limb regeneration is measured by the regeneration index [*R* index = length of limb regenerate × 100/carapace width (Bliss *et al.*, 1966)]; in land crabs, the *R* index increases from 0 to a maximum of 22–24 about one week before ecdysis (Skinner and Graham, 1970, 1972; Holland and Skinner, 1976).

Molting in decapod crustaceans is controlled by the X-organ/sinus gland complex, which is located within the eyestalks and secretes a neuropeptide, molt-inhibiting hormone (MIH), that inhibits ecdysone production by a pair of

---

Received 10 October 2001; accepted 12 March 2002.

* To whom correspondence should be addressed. E-mail: don@lamar.ColoState.edu

Abbreviations: CHH, crustacean hyperglycemic hormone; 20E, 20-hydroxyecdysone; LAF_{an}, limb autotomy factor–anecdysis; LAF_{pro}, limb autotomy factor–proecdysis; LB, limb bud; LBA, limb bud autotomy; LGIF, limb growth inhibitory factor; MIH, molt-inhibiting hormone; MLA, multiple limb autotomy.

Y-organs located in the cephalothorax (reviewed in Skinner, 1985; Hopkins, 1992; Chang *et al.*, 1993; Laufer *et al.*, 1993). Neuropeptides possessing MIH activity have been isolated from a number of crustacean species (reviewed in Chang *et al.*, 1993; Chang, 2001; De Kleijn and Van Herp, 1995; Lacombe *et al.*, 1999). MIH acts by inhibiting steroidogenesis through a high-affinity receptor in the Y-organ cell membrane (Webster, 1993) coupled to a second-messenger cascade involving cAMP and cGMP (Sedlmeier and Fenrich, 1993; Saïdi *et al.*, 1994; Spaziani *et al.*, 1999). Molting, therefore, is induced by a reduction in MIH in the hemolymph; the Y-organs, which are no longer repressed, synthesize and secrete ecdysone. Ecdysone is then hydroxylated to the active molting hormone, 20E, by 20-monooxygenase activity in peripheral tissues, such as digestive gland, testis, epidermis, and muscle (Soumoff and Skinner, 1988).

Precocious molts can be induced by eyestalk ablation, which reduces circulating MIH and results in an immediate rise in hemolymph ecdysteroids (reviewed in Skinner, 1985). Precocious molts are also induced by autotomy from five to eight walking legs; adults of the land crab *Gecarcinus lateralis* molt 6–8 weeks after multiple limb autotomy (MLA) (Skinner and Graham, 1970, 1972; Holland and Skinner, 1976; reviewed in Skinner, 1985 and Mykles, 2001). Autotomy of one or more of the primary (1°) limb regenerates (limb bud autotomy, or LBA) before a critical period interrupts proecdysis until secondary (2°) limb buds differentiate and grow to the approximate size of those lost (Holland and Skinner, 1976). On the basis of these observations, Skinner (Skinner, 1985; Skinner *et al.*, 1992) proposed that limb buds (LBs) produce two factors that control proecdysial events. Limb autotomy factor–anecdysis, or LAF_{an}, produced by 1° LBs when at least five legs are autotomized, stimulates anecdysial animals to enter proecdysis. Limb autotomy factor–proecdysis, or LAF_{pro}, produced by 2° LBs in proecdysial animals when at least one 1° LB is autotomized, inhibits proecdysial processes.

The existence of LAF_{pro} challenges the central paradigm in crustacean endocrinology that all proecdysial processes are coordinated by the eyestalk neurosecretory centers. Proecdysis is also suspended by LBA in eyestalk-ligated animals (Holland and Skinner, 1976), indicating that the action of LAF_{pro} is not mediated by MIH or other factors secreted by the X-organ/sinus gland. The mode of action of LAF_{pro} is unknown. As a MIH-like factor it may act on Y-organs by inhibiting the secretion of ecdysone. It may also act on peripheral organs (*e.g.*, epidermis, 1° LB, claw muscle) by inhibiting the action of 20E or by inhibiting the conversion of ecdysone to 20E by monooxygenase.

Here we report the initial characterization of a factor from 2° LBs, the putative LAF_{pro}, that suspends molting in *G. lateralis*. The effects of LBA on 1° LB growth and hemolymph hormone levels were determined. The stability of the molt inhibitory activity in 2° LB extracts to boiling, acetic acid, and proteinase K was also determined. Our results suggest that LAF_{pro} is a MIH-like peptide.

Materials and Methods

Animals

Adults of the land crab *Gecarcinus lateralis* were collected in Bermuda or Puerto Rico and maintained as described (Koenders *et al.*, 2002). All eight walking legs were autotomized (multiple limb autotomy, or MLA) to induce limb regeneration and molting (Skinner and Graham, 1970, 1972). The molt stage was monitored by measuring the growth of the limb bud at the location of the second walking leg on the right side (R2). The regeneration (*R*) index, which is the ratio of the LB length to carapace width expressed as a percentage, varies from zero after autotomy to 22–24 by late proecdysis (stage D_1; Holland and Skinner, 1976). Limb regenerates that formed after MLA are referred to as 1° LBs, whereas those formed after autotomy of one or more 1° LBs (limb bud autotomy, or LBA) are designated as 2° LBs.

Preparation of limb bud extracts

Seven 1° LBs were autotomized from each animal at *R* index 13–15; the R2 regenerate was left for determining the effect of LBA on 1° LB growth. Secondary LBs were autotomized at *R* index 8–10 while growth of the R2 1° LB was still inhibited. Limb buds were frozen immediately in liquid nitrogen and stored at −80°C.

Six or seven LBs from each animal were homogenized in 2 ml of ice-cold deionized water with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at medium speed until the tissue was completely homogenized (about 30 s). The *R* indices of the 1° LBs were between 13 and 15 (average wet weight: 38.85 mg/LB) and of the 2° LBs were between 8 and 10 (average wet weight: 7.43 mg/LB). The homogenates were centrifuged at 5000 × *g* at 4 °C for 15 min. The supernatant fraction was divided between three 1.5-ml Eppendorf tubes, lyophilized with a Speed-Vac centrifuge, and stored at −20 °C.

To assess the temperature stability of LAF_{pro}, lyophilized pellets containing three 2° LB equivalents were dissolved in 200 μl deionized water or 0.1 N acetic acid (pH 2.9), incubated in a boiling water bath for 15 min, and centrifuged at 5000 × *g* for 15 min. Supernatant fractions were lyophilized and stored at −20 °C; they were dissolved in 120 μl deionized water just before use. To assess the stability of LAF_{pro} to proteolytic digestion, supernatant fractions from extracts boiled in deionized water were incubated with proteinase K (Boehringer Mannheim; 0.5 μg/μl) at 50 °C for 1.5 h, lyophilized, and stored at −20 °C; they were dissolved in 120 μl deionized water just before use. The rationale for heating the extract before proteinase

K treatment was to remove contaminating proteins (LAF_{pro} was resistant to high temperatures) and therefore increase the effectiveness of the enzyme by reducing the amount of potential substrates. Secondary LB extracts from the same animal without the treatments served as positive controls.

Bioassay for inhibitory activity

The bioassay used proecdysial animals with eight 1° LBs (R index 13–16) that were growing at a constant rate (about one R index unit per day). Each animal received a series of three 40-μl injections of LB extract at 2-day intervals through the arthrodial membrane at the base of a walking leg. Initial experiments examined the effects of extracts from 1° or 2° LBs. Lyophilized pellets containing 6–7 LB equivalents were dissolved in 120 μl deionized water and used for an injection series. For the heat-, acetic acid-, and proteinase K-treated 2° LB extracts, three LB equivalents in 120 μl deionized water were used for an injection series, as it was found that one LB equivalent per injection was as effective in inhibiting 1° LB growth as two LB equivalents. The growth of the R2 1° LB was monitored before, during, and after the injection series at 2-day intervals.

Measurement of ecdysteroid by radioimmunoassay

Samples (100 μl) of hemolymph were combined with 300 μl methanol and stored at -20 °C for later analysis. Suspensions were centrifuged at $10{,}000 \times g$ for 3–5 min to remove precipitated protein. Supernatant fractions were measured for ecdysteroid by radioimmunoassay as described by Chang and O'Connor (1979). Briefly, duplicate 100-μl samples were lyophilized and dissolved in 100 μl ^{3}H-ecdysone (8000 cpm/100 μl 0.1 M sodium borate, pH 8.4). Ecdysone antiserum (Horn *et al.*, 1976) (100 μl 1:1000 dilution in 6% normal rabbit serum, 0.002% methiolate, and 0.1 M sodium borate, pH 8.4) was added to each tube and incubated at room temperature for 2–4 h. After incubation, antibody was precipitated with 200 μl saturated (100%) ammonium sulfate in 0.1 M sodium borate (pH 8.4) and centrifuged at $4800 \times g$ at 4 °C for 15 min. The pellets were washed with 400 μl 50% saturated ammonium sulfate, centrifuged at $4800 \times g$ at 4 °C for 15 min, dissolved in 25 μl deionized water, and mixed with 600 μl CytoScint scintillation cocktail (ICN Biomedicals, Inc.). Radioactivity was quantified with a Beckman LS7000 liquid scintillation counter.

Statistical analysis

Data are presented as mean ± 1 standard deviation (SD). The Student's t test was used to determine the significance of the means between control and experimental treatments, except for the data presented in Figure 6, in which the Wilcoxon matched-pairs signed-ranks test was used.

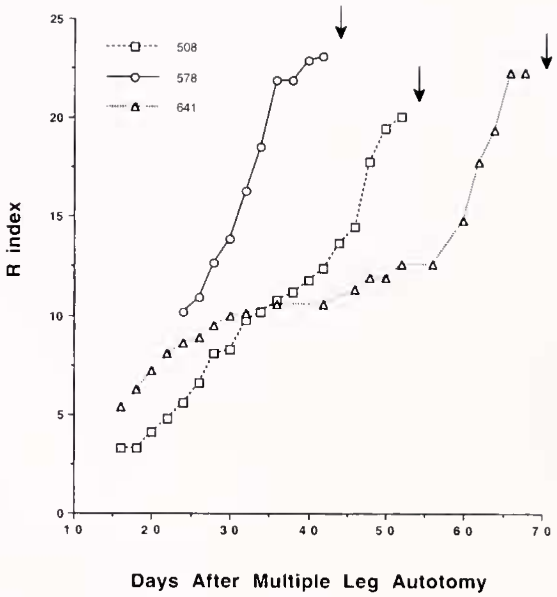

Figure 1. Primary limb bud growth and molting in *Gecarcinus lateralis* without interruption by limb bud autotomy. Animals were induced to autotomize eight walking legs on Day 0 (multiple leg autotomy, or MLA). The length of the 1° LB at the second position on the right side (R2) was measured every 2 days until molting; R indices were calculated as described in Materials and Methods. Three adult males (#508, #578, and #641) were selected to represent the range of responses to MLA. In most cases it took 30–45 days for animals to reach an R index of 15 after MLA (#508, #578). In some cases, this period could be as long as 60 days (#641). Ecdysis (arrows) occurred at 45 days (#578), 55 days (#508), and 70 days (#641) post-MLA.

Results

Effects of leg or limb bud autotomy on molting

Multiple leg autotomy induced precocious molting. The 1° LB at the second position on the right side (R2) was measured every other day after formation of basal papillae (R index 4–8). It usually took 30 to 45 days for animals to reach an R index of 15; the R index reached a maximum of 22–24 about 1 week before ecdysis. There was considerable individual variation in LB growth. Figure 1 shows the measurements from three individuals to represent the range of responses observed. Some animals responded quickly, with rapid LB growth rates and subsequent ecdysis 5 to 6 weeks after MLA (Fig. 1, animal #578). Other animals responded more slowly and molted about 10 weeks after MLA (Fig. 1, animal #641). Other animals had intermediate responses (Fig. 1, animal #508).

As shown previously (Holland and Skinner, 1976), LBA before a critical period (R index < 17) suspended LB growth and delayed molting. The measurements from one individual are shown in Figure 2 and are typical of the response of proecdysial crabs to LBA. The R2 1° LB stopped growing when the other seven 1° LBs were autotomized. The 2° LB

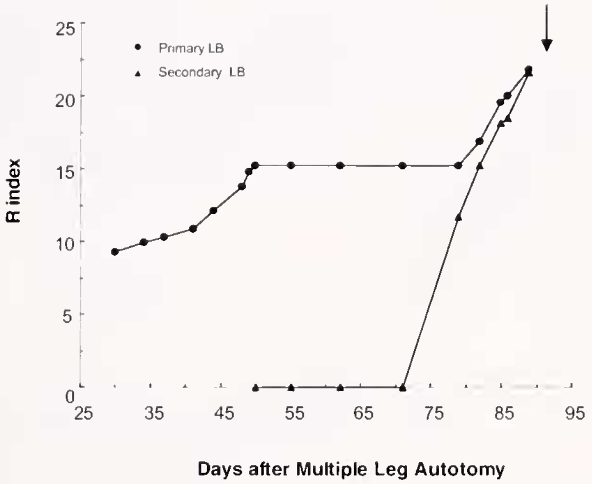

Figure 2. Limb bud autotomy suspends molting. All eight walking legs were autotomized from an adult male at Day 0, and the growth of the R2 1° LB was measured (●). On Day 50 (*R* index 15), all but the R2 1° LB were autotomized, and the growth of the L2 2° LB was measured (▲). LBA suspended proecdysis until the 2° LBs differentiated and grew to about the same size as the R2 1° LB that remained. The animal molted 91 days post-MLA (arrow), delaying ecdysis about 4 weeks.

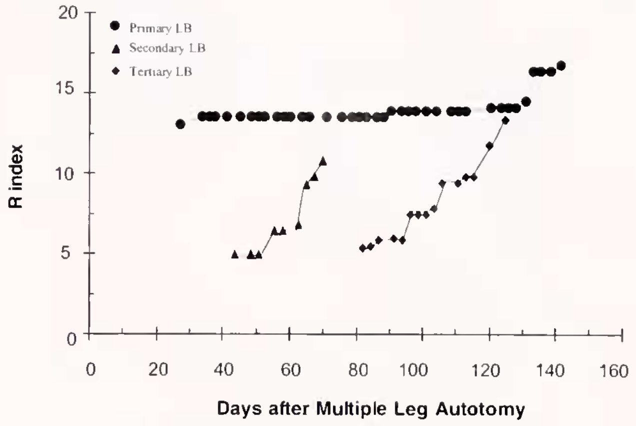

Figure 3. Autotomy of secondary limb buds prolongs proecdysis. Eight walking legs were autotomized from an adult male at Day 0, and the growth of the R2 1° LB was measured (●). On Day 27 (*R* index 12.5), seven 1° LBs were autotomized, and the growth of the L2 2° LB was measured (▲). On Day 70 all seven of the 2° LBs were autotomized, and the growth of the L2 3° LB was measured (◆). Growth of the R2 1° LB was suspended until the 3° LBs grew to a size similar to that of the 1° LB. The crab died 145 days post-MLA, before molting.

at the second position on the left side (L2) was used to monitor 2° LB growth. Primary LB growth remained inhibited until the L2 2° LB reached a size similar to that of the autotomized 1° LB; subsequently, both 1° and 2° LBs grew continuously (Fig. 2). A consequence of LBA was that molting was delayed several weeks; for the data from the animal shown in Figure 2, molting was delayed about 30 days.

Autotomy of 2° LBs prolonged molting even further. In the experiment shown in Figure 3, seven 1° LBs were autotomized at *R* index 12.5 twenty-five days after MLA. On Day 70, all seven 2° LBs were autotomized; tertiary (3°) LBs differentiated and grew. Growth of the 1° LB remained inhibited until the L2 3° LB attained the size of the 1° LB. It took longer for the 3° LB to grow to a size similar to that of the 2° LB that was autotomized at the same location.

Effects of limb bud autotomy on hemolymph ecdysteroid

LBA reduced ecdysteroid levels in the hemolymph of animals induced to molt by MLA. In a control group in which molting was not interrupted by LBA, ecdysteroid levels increased continuously during proecdysis (Fig. 4, filled circles). In another group of animals, LBs were autotomized at *R* index 13–16, and hemolymph was sampled either during inhibition or after resumption of 1° LB growth (Fig. 4, filled and open triangles, respectively). The average ecdysteroid concentration in inhibited animals (130.3 ± 16.2 ng/ml; $n = 24$) was 45% that of the control animals (287.7 ± 28.3 ng/ml; $n = 30$) within the same range of *R* indices (between 14 and 18 in Fig. 4). However, due to the large amount of variation between individuals, the means were not significantly different. The ecdysteroid levels of animals after resumption of 1° LB growth fell within those of the control group.

Since ecdysteroid titers varied between individuals, sam-

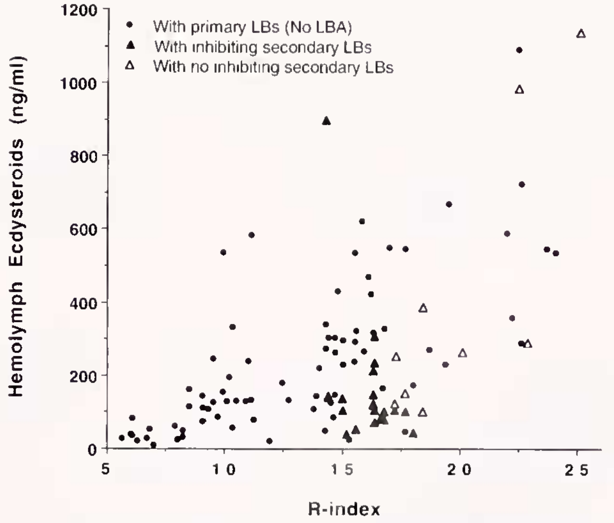

Figure 4. Effect of limb bud autotomy on hemolymph ecdysteroid concentrations during proecdysis. Molting was induced by autotomy of all eight walking legs, and 1° LB growth was monitored (*R* index). Samples were taken from three groups of animals: a control group in which proecdysis was not interrupted by LBA (●); a group subjected to LBA at *R* index 13–16 with 2° LBs (*R* index 8–10) inhibiting 1° LB growth (▲); and a group subjected to LBA at *R* index 13–16 with 2° LBs (*R* index >16) no longer inhibiting 1° LB growth (△). In most cases, hemolymph ecdysteroid was lower in animals in which 2° LBs inhibited 1° LB growth.

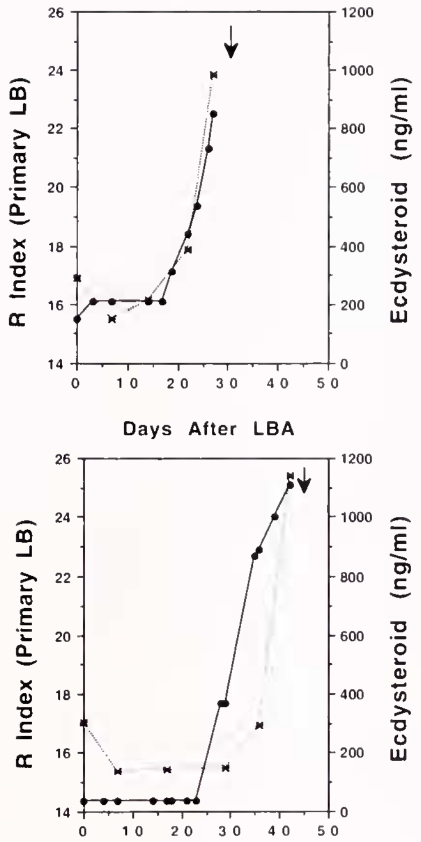

Figure 5. Effect of limb bud autotomy on hemolymph ecdysteroid concentrations in two individual crabs during proecdysis. Eight walking legs were autotomized, and the growth of the R2 1° LB was measured and expressed as *R* index (●). On Day 0, all but the R2 1° LB were autotomized, and hemolymph samples were taken at various intervals post-LBA. The ecdysteroid concentration (X) dropped within one week after LBA and remained low until the L2 2° LB approached the size of the contralateral 1° LB. There was no consistent relationship between resumption of 1° LB growth and ecdysteroid levels. For animal #805 (lower panel), the 1° LB resumed growth at Day 29 post-LBA, even though ecdysteroid level did not increase until Day 36. For animal #771 (upper panel), hemolymph ecdysteroid began to increase at Day 14, but 1° LB growth did not resume until Day 19. Arrows indicate when ecdysis occurred.

ples were taken from the same individuals on the day of LBA and the days following LBA. In Figure 5, hemolymph ecdysteroid concentrations and 1° LB growth (*R* index) from two animals (#805 and #771) are presented. LBA resulted in an immediate cessation of 1° LB growth, and ecdysteroid levels decreased about 52% by one week post-LBA. Levels remained low until 1° LB growth resumed, and the animals eventually molted. However, resumption of 1° LB growth did not always follow an increase in ecdysteroid concentration. For animal #805 (Fig. 5, upper panel), the 1° LB resumed growth by Day 29 post-LBA, even though ecdysteroid level did not increase until Day 36. For animal #771, however, hemolymph ecdysteroid began to increase by Day 14, but 1° LB growth did not resume until Day 19 (Fig. 5, lower panel). Ecdysteroid reached a maximum concentration of more than 1000 ng/ml just before ecdysis. Data from a group of animals in which hemolymph samples were taken on the day of LBA and one week after LBA are summarized in Figure 6. The level was reduced 73% at one week post-LBA; the means are significantly different ($P < 0.043$, using the Wilcoxon matched-pairs signed-ranks test).

Effects of primary and secondary limb bud extracts on molting

Proecdysial animals (*R* index 13–15) with continuously growing 1° LBs were selected for determining the effects of LB extracts on molting. An injection series consisted of injections of either 1° or 2° LB extracts (two LB equivalents/injection) at 2-day intervals, for a total of three injections per animal. Twelve proecdysial crabs were injected with 2° LB extracts; the data from three animals are shown in Figure 7 (open symbols) to represent the range of responses observed. Secondary LB extracts inhibited the growth of 1° LB during the injection series. This effect was usually sustained 2–4 days after the last injection before 1° LB growth resumed (Fig. 7, animals #124 and #386). In contrast, injection of 1° LB extracts had no effect on 1° LB growth. The data from one animal (Fig. 7; #331) is representative of the results from ten proecdysial animals that were injected with 1° LB extracts.

To quantify the effects of LB extracts, the 1° LB growth rates were determined for the 1-week intervals before and after the first injection of the series (Fig. 8). Secondary LB extracts significantly inhibited 1° LB growth by 68%, from 1.08 *R* index/day before injection to 0.35 *R* index/day dur-

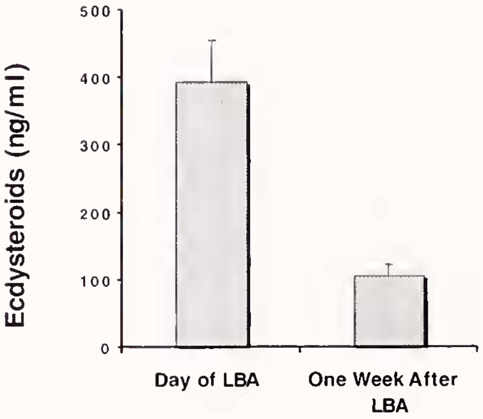

Figure 6. Hemolymph ecdysteroid concentrations before and after limb bud autotomy. Eight walking legs were autotomized, and the growth of the R2 1° LB was measured. On Day 0, a hemolymph sample was taken, followed by autotomy of all 1° LBs except the R2 1° LB, on Day 7, a second hemolymph sample was taken. LBA reduced significantly the mean ecdysteroid level 73% by one week post-LBA ($P < 0.043$; $n = 5$). Error bars are ± 1 SD.

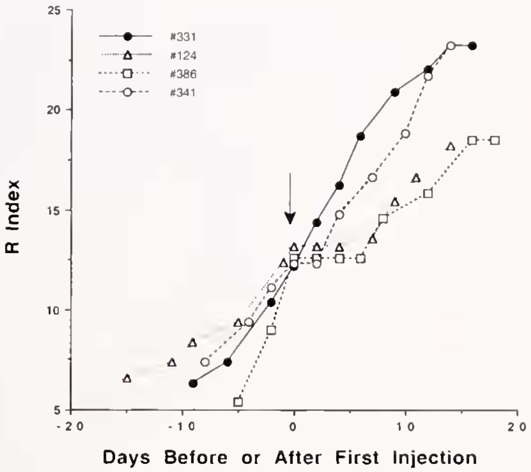

Figure 7. Effects of injection of limb bud extracts on 1° limb bud growth. Proecdysial animals (*R* index 13–15) were injected with extracts of either 1° LBs (●) or 2° LBs (open symbols) three times at 2-day intervals beginning at Day 0 (arrow). Each injection contained two LB equivalents (see Materials and Methods). The effect of injections was determined by monitoring the *R* index of the R2 1° LB. The injection of 2° LB extracts inhibited 1° LB growth (animals #124, #386, and #341), whereas injection of 1° LB extract had no effect (#331).

ing injection ($P < 0.005$; $n = 12$). The 1° LB growth rates of animals injected with 1° LB extracts were the same before and during the injection series ($P < 0.86$; $n = 10$). For animals that were allowed to complete proecdysis and molt, injection of 2° LB extract delayed ecdysis about 6 days. The interval between the first injection to ecdysis was 16 to 18 days for three animals injected with 1° LB extract and 22 to 24 days for three animals injected with 2° LB extract.

Characterization of limb autotomy factor–proecdysis

The reduction in hemolymph ecdysteroid concentration after LBA suggested that $\mathrm{LAF_{pro}}$, like MIH, inhibits ecdysteroid secretion by the Y-organs. Consequently, we determined whether $\mathrm{LAF_{pro}}$ shared physical and chemical properties with MIH. MIH is a neuropeptide that is resistant to boiling in deionized water or acids, but is inactivated by proteases (Ranga Rao, 1965; Freeman and Costlow, 1979; Webster, 1986; Webster and Keller, 1986; Chang *et al.*, 1990).

To determine the properties of $\mathrm{LAF_{pro}}$, 2° LB extracts were either boiled in deionized water, boiled in 0.1 N acetic acid, or incubated with proteinase K. An injection series consisted of three injections at 2-day intervals; each injection contained one LB equivalent (because we found that one LB equivalent was as effective as two, we used one LB equivalent to conserve material). The growth of the R2 1° LB was measured for the week before and the week after the first injection. The thermal stability of $\mathrm{LAF_{pro}}$ was determined by heating 2° LB extracts in a boiling water bath for 15 min. The denatured protein was removed by centrifugation, and the supernatant fraction was injected into proecdysial animals. The boiled extract significantly inhibited 1° LB growth by 68%, indicating that the factor was heat-stable (Fig. 9). In a second set of experiments, the injection of extracts boiled in 0.1 N acetic acid had no effect on 1° LB growth, while the positive control (2° LB extracts from the same animal boiled 15 min in deionized water) inhibited growth (Fig. 9). In a third set of experiments, incubation of 2° LB extracts with proteinase K also destroyed $\mathrm{LAF_{pro}}$ activity, as injection of treated extracts did not inhibit 1° LB growth (Fig. 9). Positive controls, in which untreated 2° LB extracts from the same animals were injected, inhibited 1° LB growth (data not shown).

Discussion

Since LB growth in decapod crustaceans is restricted to the proecdysial stage, limb regeneration must be coordinated and integrated with other physiological processes for an animal to molt successfully. In various decapod species, multiple leg autotomy (MLA) acts as a potent inducer of precocious molting, probably due to the need to restore functional appendages as quickly as possible (Skinner and Graham, 1970, 1972; Holland and Skinner, 1976; see Skinner, 1985, for additional references). Limb bud autotomy (LBA) can delay molting so that animals will molt with a

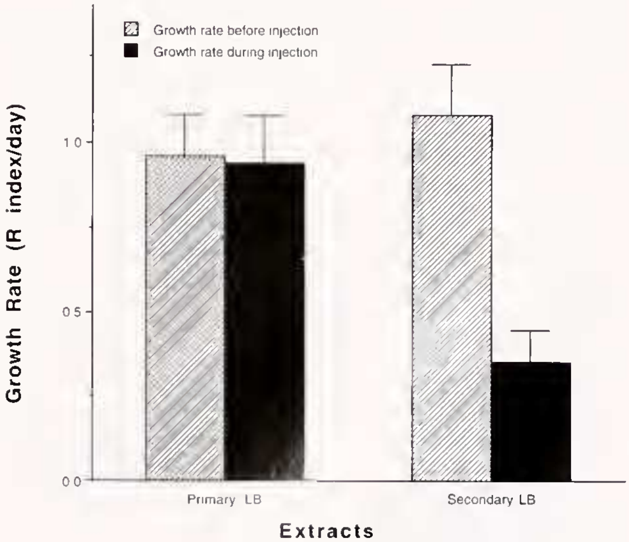

Figure 8. Injection of 2° limb bud extracts inhibits 1° LB growth. Proecdysial animals were injected with extracts of either 1° or 2° LBs, and the effect of injections was determined by monitoring the *R* index of the R2 1° LB. The graph compares the mean 1° LB growth rates the week before and the week after the first injection. Secondary LB extract significantly inhibited 1° LB growth ($P < 0.05$; $n = 12$). Primary LB extract did not have any significant effect on 1° LB growth ($P < 0.86$, $n = 10$). Error bars are ± 1 SD.

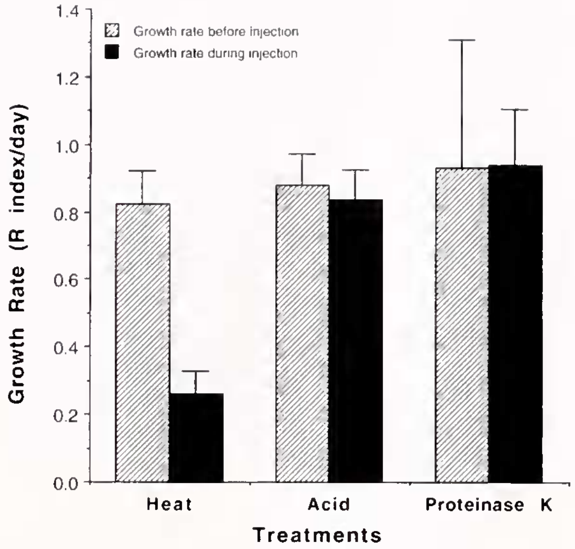

Figure 9. Characterization of LAF$_{pro}$ activity in 2° limb bud extracts. Extracts of 2° LBs were first either boiled in deionized water, boiled in 0.1 N acetic acid, or incubated with proteinase K; they were injected into proecdysial animals (*R* index 13–15; see Materials and Methods). The effects of injections were determined by comparing the mean 1° LB growth rates during the week before and the week after the first injection. Each injection contained one LB equivalent. Extracts boiled in deionized water retained inhibitory activity; growth rate was inhibited 68% ($P < 0.05, n = 10$). However, boiling in 0.1 N acetic acid or incubation with proteinase K destroyed the inhibitory activity; growth rates were not significantly different ($P < 0.37, n = 8$ for acetic acid and $P < 1.78, n = 7$ for proteinase K). Error bars are ± 1 SD.

full complement of appendages (Holland and Skinner, 1976; Weis, 1976; McConaugha, 1991). In *Gecarcinus lateralis*, Holland and Skinner (1976) showed that autotomy of one or more LBs before a critical stage in proecdysis (*R* index < 17) delays ecdysis several weeks so that 2° LBs are regenerated before the next molt. LBA causes slowing (autotomy at *R* index 7–10) or complete cessation (autotomy at *R* index 10–16) of growth of any remaining 1° LBs, as measured by *R* index and DNA synthesis, until 2° LBs differentiate and grow to about the same size as the 1° LBs that remain (Holland and Skinner, 1976). We obtained similar results in this study (Fig. 2). Furthermore, we showed that proecdysis is prolonged further by autotomy of 2° LBs (Fig. 3). Growth of the 3° LBs is slower than that of the 2° LBs, which suggests that repetitive regeneration places increasing energetic demands on an animal. Such demands can limit the number or size of regenerates or reduce the size increment at ecdysis (Chittleborough, 1975; Kuris and Mager, 1975; McConaugha, 1991).

The hormone 20E plays a central role in regulating molting. Thus, it is not surprising that LBA lowers hemolymph ecdysteroid levels. The changes in ecdysteroid concentration over the molt cycle follow the same general pattern in all decapod species. Beginning at proecdysis, decreased secretion of MIH results in derepression of the Y-organs. Ecdysteroid levels increase during proecdysis to a maximum in stage D$_3$, drop precipitously at stage D$_4$, and remain at low levels through metecdysis and anecdysis (reviewed in Skinner, 1985). Circulating ecdysteroid drops in response to LBA and remains low during basal regeneration; ecdysteroid titers begin to increase during the subsequent growth of the 2° LB (Figs. 5, 6; McCarthy and Skinner, 1977). Primary LBs removed later in proecdysis (*R* index > 17) are not regenerated, and the animal molts without the full complement of appendages (Holland and Skinner, 1976). In this case hemolymph ecdysteroid levels remain elevated (Fig. 4). This may result from reduced release of LAF$_{pro}$ from 2° LBs. Alternatively, if LAF$_{pro}$ release is sustained at *R* index >17, then elevated ecdysteroid may result from reduced sensitivity of Y-organs to LAF$_{pro}$. This is analogous to the reduced sensitivity of Y-organs to MIH during late proecdysis when there is maximal ecdysone production and high levels of ecdysteroid in hemolymph (Blais *et al.*, 1994).

Primary and 2° LBs respond differently to the same concentration of ecdysteroid, which suggests that limb regeneration is not regulated simply by ecdysteroid levels. It probably involves interactions between ecdysteroid and peptide factors on tissues that may have different sensitivities to these factors. Low ecdysteroid allows formation of basal papillae, since injection of exogenous ecdysone inhibits LB differentiation (Hopkins *et al.*, 1979). Higher levels are required for sustained proecdysial LB growth (Hopkins, 1989). LBA causes a decline in ecdysteroid, but the levels are still higher than those of anecdysial and early proecdysial animals (Figs. 4–6). At these levels following LBA, 2° LBs differentiate and grow, whereas 1° LBs stop growing. This differential sensitivity may be due to a higher expression of ecdysteroid receptors in 2° LBs, thus causing the 2° LBs to respond as if the ecdysteroid concentration were higher. In *Uca pugilator*, ecdysteroid receptor mRNA levels increase in 1° LBs during the proecdysial growth phase, but receptor expression was not examined in 2° LBs (Chung *et al.*, 1998). Furthermore, resumption of LB growth is not always preceded with increases in hemolymph ecdysteroid (Fig. 5), suggesting that other factors are involved. One possible factor is a limb growth-inhibiting factor (LGIF), which may be secreted by eyestalk neurosecretory centers to slow LB growth in *G. lateralis* (Hopkins *et al.*, 1979).

Secondary LBs contain a factor that inhibits proecdysial growth of limb buds and delays molting (Figs. 7, 8). We believe this factor is LAF$_{pro}$, the existence of which was originally proposed by Skinner (1985). Extracts of 2° LBs were injected into proecdysial animals before (*R* index 13–15) or near (*R* index 16–17) the critical period. Previous work by Holland and Skinner (1976) suggested that animals near the critical period would be less responsive to the extract. As expected, growth of 1° LBs slowed in the animals near the critical period (data not shown), but was completely blocked in animals at *R* index 13–15. Limb bud growth resumed 3–5 days after the final injection. Since injection of 1° LB extracts did not inhibit LB growth, it is unlikely that the cessation of LB growth by 2° LB extracts was a consequence of the injection procedure or a general toxic reaction.

Initial characterization of LAF_{pro} indicates that it is a novel molt-inhibitory factor. It is a small peptide, as it is not denatured when boiled in deionized water and it is degraded by proteinase K (Fig. 9). However, LAF_{pro} is inactivated in 0.1 N acetic acid, whereas other inhibitory peptides, such as MIH and LGIF, are acid-stable (Ranga Rao, 1965; Freeman and Costlow, 1979; Hopkins *et al.*, 1979; Webster, 1986; Webster and Keller, 1986; Chang *et al.*, 1990). MIH is a neuropeptide of about 8.5 kDa (71–78 amino acid residues, depending on species) that inhibits synthesis and secretion of ecdysone by the Y-organs (reviewed in Chang *et al.*, 1993; Chang, 2001; De Kleijn and Van Herp, 1995; Lacombe *et al.*, 1999). LGIF (M_r approximately 1 kDa) inhibits LB growth in *G. lateralis* at concentrations that have no effect on molting, and thus it appears to be distinct from MIH (Hopkins *et al.*, 1979).

Although LAF_{pro} appears to be a small peptide related to MIH, its identity remains incomplete until the inhibitory factor is purified and sequenced. Those efforts are underway. The site of LAF_{pro} synthesis is also unknown, but the factor may be synthesized in neurons in the thoracic ganglion or nerve roots and transported *via* axonal processes to the 2° LBs. This is supported by the recent discovery of crustacean hyperglycemic hormone (CHH)/MIH-immunoreactive neurons in the thoracic second roots in lobster (Chang *et al.*, 1999). Cells containing CHH-like peptides are also found in the pericardial organ and gastrointestinal tract of *Carcinus maenas* (Chung *et al.*, 1999; Webster *et al.*, 2000; Dircksen *et al.*, 2001). In lobster, hemolymph levels of CHH/MIH drop 20 days after eyestalk ablation, although significant amounts are detected as long as one year without eyestalks (Chang *et al.*, 1998). These data indicate that extra-eyestalk sites for neuropeptide synthesis and secretion are common and are biologically important. Perhaps the most compelling evidence for an extra-LB site of LAF_{pro} synthesis is that LBA has an immediate inhibition on 1° LB growth before any 2° LBs differentiate (Fig. 2; Holland and Skinner, 1976). The tissue target (or targets) of LAF_{pro} also needs to be identified. All the data are consistent with the view that LAF_{pro} acts directly on the Y-organs by inhibiting ecdysone synthesis and secretion. The neurosecretory centers in the eyestalks are not involved, since LBA inhibits 1° LB growth and lowers hemolymph ecdysteroid in eyestalk-ablated animals (Holland and Skinner, 1976; McCarthy and Skinner, 1977). We have begun experiments to determine whether LAF_{pro} blocks ecdysone secretion by land crab Y-organs *in vitro*. Our working model is that severing the thoracic nerve by LBA results in LAF_{pro} transport from neurosecretory cells in the central nervous system and release of LAF_{pro} into the hemolymph at the autotomy plane (reviewed in Mykles, 2001). LAF_{pro} suppresses ecdysone secretion by Y-organs, thus inhibiting 1° LB growth and other proecdysial processes that are ecdysteroid-dependent.

Acknowledgments

This research was supported by the National Science Foundation (IBN-9723576 and IBN-9904528). We thank Bill Mitchell (Bermuda Aquarium and Zoo), Hector C. Horta (Puerto Rico Department of Natural Resources), Hector J. Horta, and Vicki and Christopher Mykles for collecting land crabs; Courtney Rocheleau, Phillip Mendoza, and Laura Baker (Colorado State University) for animal care and technical assistance; and Eva Mulder and Sharon Chang (Bodega Marine Laboratory) for technical assistance. The authors acknowledge Dr. Dorothy M. Skinner, whose pioneering work served as inspiration to this research.

Literature Cited

Blais, C., M. Sefiani, J.-Y. Toullec, and D. Soyez. 1994. *In vitro* production of ecdysteroids by Y-organs of *Penaeus vannamei* (Crustacea, Decapoda). Correlation with hemolymph titers. *Invertebr. Reprod. Dev.* **26:** 3–11.

Bliss, D. E., S. M. E. Wang, and E. A. Martinez. 1966. Water balance in the land crab, *Gecarcinus lateralis*, during the intermolt cycle. *Am. Zool.* **6:** 197–212.

Chang, E. S. 2001. Crustacean hyperglycemic hormone family: old paradigms and new perspectives. *Am. Zool.* **41:** 380–388.

Chang, E. S., and J. D. O'Connor. 1979. Arthropod molting hormones. Pp. 797–814 in *Methods of Hormone Radioimmunoassay*, B. M. Jaffe and H. R. Behrman, eds. Academic Press, New York.

Chang, E. S., G. D. Prestwich, and M. J. Bruce. 1990. Amino acid sequence of a peptide with both molt-inhibiting and hyperglycemic activities in the lobster, *Homarus americanus*. *Biochem. Biophys. Res. Commun.* **171:** 818–826.

Chang, E. S., M. J. Bruce, and S. L. Tamone. 1993. Regulation of crustacean molting: a multi-hormonal system. *Am. Zool.* **33:** 324–329.

Chang, E. S., R. Keller, and S. A. Chang. 1998. Quantification of crustacean hyperglycemic hormone by ELISA in hemolymph of the lobster, *Homarus americanus*, following various stresses. *Gen. Comp. Endocrinol.* **111:** 359–366.

Chang, E. S., S. A. Chang, B. S. Beltz, and E. A. Kravitz. 1999. Crustacean hyperglycemic hormone in the lobster nervous system: localization and release from cells in the subesophageal ganglion and thoracic second roots. *J. Comp. Neurol.* **414:** 50–56.

Chittleborough, R. G. 1975. Environmental factors affecting growth and survival of juvenile western rock lobsters *Panulirus longipes* (Milne-Edwards). *Aust. J. Mar. Freshw. Res.* **26:** 117–196.

Chung, A. C. K., D. S. Durica, and P. M. Hopkins. 1998. Tissue-specific patterns and steady-state concentrations of ecdysteroid receptor and retinoid-X-receptor mRNA during the molt cycle of the fiddler crab, *Uca pugilator*. *Gen. Comp. Endocrinol.* **109:** 375–389.

Chung, J. S., H. Dircksen, and S. G. Webster. 1999. A remarkable, precisely timed release of hyperglycemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas*. *Proc. Natl. Acad. Sci. USA* **96:** 13,103–13,107.

De Kleijn, D. P. V., and F. Van Herp. 1995. Molecular biology of neurohormone precursors in the eyestalk of Crustacea. *Comp. Biochem. Physiol.* **112B:** 573–579.

Dircksen, H., D. Böcking, U. Heyn, C. Mandel, J. S. Chung, G. Baggerman, P. Verhaert, S. Daufeldt, T. Plösch, P. P. Jaros, E. Waelkens, R. Keller, and S. G. Webster. 2001. Crustacean hyperglycaemic hormone (CHH)-like peptides and CHH-precursor-related peptides from pericardial organ neurosecretory cells in the shore crab, *Carcinus maenas,* are putatively spliced and modified products of multiple genes. *Biochem. J.* **356:** 159–170.

Freeman, J. A., and J. D. Costlow. 1979. Hormonal control of apolysis in barnacle mantle tissue epidermis, *in vitro. J. Exp. Zool.* **210:** 333–346.

Holland, C. A., and D. M. Skinner. 1976. Interactions between molting and regeneration in the land crab. *Biol. Bull.* **150:** 222–240.

Hopkins, P. M. 1989. Ecdysteroids and regeneration in the fiddler crab *Uca pugilator. J. Exp. Zool.* **252:** 293–299.

Hopkins, P. M. 1992. Hormonal control of the molt cycle in the fiddler crab *Uca pugilator. Am. Zool.* **32:** 450–458.

Hopkins, P. M. 1993. Regeneration of walking legs in the fiddler crab *Uca pugilator. Am. Zool.* **33:** 348–356.

Hopkins, P. M. 2001. Limb regeneration in the fiddler crab *Uca pugilator*: hormonal and growth factor control. *Am. Zool.* **41:** 389–398.

Hopkins, P. M., D. E. Bliss, S. W. Sheehan, and J. R. Boyer. 1979. Limb growth-controlling factors in the crab *Gecarcinus lateralis,* with special reference to the limb growth-inhibiting factor. *Gen. Comp. Endocrinol.* **39:** 192–207.

Horn, D. H. S., J. S. Wilkie, B. A. Sage, and J. D. O'Connor. 1976. A high affinity antiserum specific for the ecdysone nucleus. *J. Insect Physiol.* **22:** 901–905.

Koenders, A., X. L. Yu, E. S. Chang, and D. L. Mykles. 2002. Ubiquitin and actin expression in claw muscles of land crab, *Gecarcinus lateralis,* and American lobster, *Homarus americanus*: differential expression of ubiquitin in two slow muscle fiber types during molt-induced atrophy. *J. Exp. Zool.* **292:** 618–632.

Kuris, A. M., and M. Mager. 1975. Effect of limb regeneration on size increase at molt of the shore crabs *Hemigrapsus oregonensis* and *Pachygrapsus crassipes. J. Exp. Zool.* **193:** 353–360.

Lacombe, C., P. Grève, and G. Martin. 1999. Overview on the subgrouping of the crustacean hyperglycemic hormone family. *Neuropeptides* **33:** 71–80.

Laufer, H., J. S. B. Ahl, and A. Sagi. 1993. The role of juvenile hormones in crustacean reproduction. *Am. Zool.* **33:** 365–374.

McCarthy, J. F., and D. M. Skinner. 1977. Interruption of proecdysis by autotomy of partially regenerated limbs in the land crab, *Gecarcinus lateralis. Dev. Biol.* **61:** 299–310.

McConaugha, J. R. 1991. Limb regeneration in juveniles of the mud crab *Rhithropanopeus harrisii* following removal of developing limb buds. *J. Exp. Zool.* **257:** 64–69.

Mykles, D. L. 2001. Interactions between limb regeneration and molting in decapod crustaceans. *Am. Zool.* **41:** 399–406.

Ranga Rao, K. 1965. Isolation and partial characterization of the molt-inhibiting hormone of the crustacean eyestalk. *Experientia* **21:** 593–594.

Saïdi, B., N. De Bessé, S. G. Webster, D. Sedlmeier, and F. Lachaise. 1994. Involvement of cAMP and cGMP in the mode of action of molt-inhibiting hormone (MIH), a neuropeptide which inhibits steroidogenesis in a crab. *Mol. Cell. Endocrinol.* **102:** 53–61.

Sedlmeier, D., and R. Fenrich. 1993. Regulation of ecdysteroid biosynthesis in crayfish Y-organs: I. Role of cyclic nucleotides. *J. Exp. Zool.* **265:** 448–453.

Skinner, D. M. 1985. Molting and regeneration. Pp. 43–146 in *The Biology of Crustacea,* D. E. Bliss and L. H. Mantel, eds. Academic Press, New York.

Skinner, D. M., and D. E. Graham. 1970. Molting in land crabs: stimulation by leg removal. *Science* **169:** 383–385.

Skinner, D. M., and D. E. Graham. 1972. Loss of limbs as a stimulus to ecdysis in Brachyura (true crabs). *Biol. Bull.* **143:** 222–233.

Skinner, D. M., S. S. Kumari, and J. J. O'Brien. 1992. Proteins of the crustacean exoskeleton. *Am. Zool.* **32:** 470–484.

Soumoff, C., and D. M. Skinner. 1988. Ecdysone 20-monooxygenase activity in land crabs. *Comp. Biochem. Physiol.* **91C:** 139–144.

Spaziani, E., M. P. Mattson, W. N. L. Wang, and H. E. McDougall. 1999. Signaling pathways for ecdysteroid hormone synthesis in crustacean Y-organs. *Am. Zool.* **39:** 496–512.

Webster, S. G. 1986. Neurohormonal control of ecdysteroid biosynthesis by *Carcinus maenas* Y-organs *in vitro,* and preliminary characterization of the putative molt-inhibiting hormone (MIH). *Gen. Comp. Endocrinol.* **61:** 237–247.

Webster, S. G. 1993. High-affinity binding of putative moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) to membrane-bound receptors on the Y-organ of the shore crab *Carcinus maenus. Proc. R. Soc. Lond.* **251:** 53–59.

Webster, S. G., and R. Keller. 1986. Purification, characterisation and amino acid composition of the putative moult-inhibiting hormone (MIH) of *Carcinus maenas* (Crustacea, Decapoda). *J. Comp. Physiol.* **156:** 617–624.

Webster, S. G., H. Dircksen, and J. S. Chung. 2000. Endocrine cells in the gut of the shore crab *Carcinus maenas* immunoreactive to crustacean hyperglycaemic hormone and its precursor-related peptide. *Cell Tissue Res.* **300:** 193–205.

Weis, J. S. 1976. Regeneration in the fiddler crab, *Uca pugilator,* after injury or removal of limb buds. *J. Exp. Zool.* **197:** 21–30.